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To whom it may concern.

Enclosed, please find in support of 10/076,266 patent Application (Confirmation # 1857, Filing Receipt no: 0C0000000 793 8363), a copy of my manuscript that was accepted for publication in "The International Journal of Experimental Pathology" (London, UK) on 6/27/2002.

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Thank you for your attention to this matter.

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Denis K. Solomon.



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THE INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY IS
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This paper was accepted for publication on 6/27/2002.

It is a reworking of the manuscript entitled 'The inter-relationships between human endothelial cell secretions etc.'

This manuscript is being sent to you in support of patent application no. 10/076,266
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An in vitro examination of an extracellular matrix scaffold for
use in wound healing.

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Summary This paper provides evidence that an extracellular matrix (ECM) secreted by human umbilical vein endothelial cells (HUVECs)/gelatin assembly overlaid by a mixed matrix secreted by human dermal microvascular endothelial cells (HDMECs) and human dermal fibroblasts provides a viable acellular scaffold for use in wound healing.

Trypsinized epidermal keratinocytes or colonies from Dispase-digested fresh and cadaver skin tissue adhered and proliferated on either HUVECs ECM/gelatin or mixed matrix overlaid on HUVECs ECM/gelatin. An epithelial-mesenchymal interaction, previously thought to be tissue specific was exposed as well as concomitant integrin versatility. Furthermore, heterologous HDMECs and dermal fibroblasts attached and proliferated on the mixed matrix as well as HUVECs ECM.

The conditioned medium from HUVECs (HUVECs CM) was found to

neutralize the lingering after effects of Dispace, and could be used for the tissue culture of epidermal keratinocytes, HDMECs and dermal fibroblasts, which share related extracellular secretions.

Taken together, these results indicate that cultured epithelial autografts can be redesigned to include both epithelial and dermal elements, and advances the acellular 'sandwich' ECM scaffold as a possible structural replacement for the lamina densa and lamina lucida, damaged or completely missing in some wounds and burns.

Keywords: human skin cell secretions, extracellular matrix scaffold, keratinocyte adhesion, integrin versatility, cultured epithelial autografts, wound healing.

Introduction

The matrix underlying vascular endothelial cells (ECM) is similar in organization and macromolecular composition to basement membranes. The macromolecular composition of endothelial cell matrices (ECMs) reportedly include collagen types I, III, IV, V, VI, fibronectin, laminin, heparan sulfate and dermatan sulfate/chondroitin sulfate proteoglycans, entactin, elastin with sequestered basic fibroblast growth factor (b-FGF), plasminogen activator and heparanase activity (Gospodarowicz et al. 1980a; Korner et al. 1993).

Human umbilical vein endothelial cells maintained on the ECM need b-FGF to become confluent (Gospodarowicz et al. 1980b), and HUVECs seeded on HUVECs ECM also need an endothelial cell growth supplement to reach confluence (Solomon 1992). Of note is the report by Yamamoto et al. (1994) that b-FGF suppressed t PA release from cultured HUVECs but enhanced that from HAECs.

In unwounded human skin, basal keratinocytes are separated from type IV collagen-rich lamina densa and interstitial collagens by a 35-nm space, the lamina lucida rich in laminin (Woodley et al. 1988). It must be borne in mind that keratinocyte adhesion and migration is significantly regulated by ECM components, such as collagens, fibronectin and laminin. It is now widely accepted that the principal basement membrane components laminin and collagen type IV, exist in various isoforms each of which is a heterotrimer of different subunit polypeptides. The activation state of heterodimeric integrin cell surface receptors is supposedly regulated by 'inside' out signals and when bound to a ligand transmit different signals to the cell interior (so-called 'outside-in' signaling) which regulate the organization of the cytoskeleton, the cell cycle and gene

expression. The structural basis of integrin activation and signaling are as yet unknown (private communication, Prof. A. Arnaout, Harvard Medical School).

Binding of integrins to the ECM stimulates intracellular signalling through integrin clustering and the formation of focal adhesion sites. Because these integrins are the direct contact between cells and the ECM, they might be important cellular mechanotransducers, and are thought to determine the organization of epidermal tissue into proliferating and differentiating compartments (Rennekampff et al 1996).

In wound healing, the explanation for the final collagen deposition in the form of scar tissue is related to tissue bleeding. Fibrin of the blood clot serves as a substrate for the attachment and in-growth of cells, mainly dermal fibroblasts. By this mechanism, the original unstable fibrin clot or mechanically weak fibrin adhesions, are turned (i.e. collagenized) into permanent fibrotic structures by the activity of invading dermal fibroblasts. Interference with the above procedure by packing the wound bed with an acellular scaffold might lead to the formation of lesser amounts of cell-rich granulation tissue and later, less cell-poor scar tissue.

Present day bioengineered skin substitutes (for a review, see Morgan & Yarmush (1997)) have their limitations and normally need an autograft or allograft eventually. Problems arise with this traditional treatment because the graft may not 'take' and grafts rarely attain the sensory qualities of normal skin.

In 1992, I reported a linkage between the acellular sub-endothelial matrix or ECM of HUVECs and that of human aortic endothelial cells (HAECs). One of the intriguing aspects of that study was the adherence of 11th passage HUVECs (isolated from one umbilical cord) on to the prepared ECM of 6th passage HUVECs, isolated from another

cord. Most of the seeded cells adhered within two hours, reached confluence in eighteen hours, and were positively characterized as endothelial cells. A postulate was proposed that HUVECs ECM shared a common phenotype and should be structurally related to HAECs ECM (Solomon 1992). That result indicated that heterologous cells could attach and proliferate on any secreted ECM from that particular cell type.

A search of the scientific literature cross-referencing several medical research disciplines was undertaken for proposed studies on dermal wound healing and uncovered further extracellular cross-relationships. Consider the table below:

Human endothelial cells	Extracellular Secretions	References
Aortic, adult vena cava	Type IV procollagen, thrombospondin and fibronectin.	Sage and Bornstein 1982; Fry et al. 1984
Umbilical vein	Type IV procollagen, thrombospondin and fibronectin.	Sage and Bornstein 1982; Fry et al. 1984
Neonatal/Adult dermis	Type IV procollagen, thrombospondin, fibronectin and laminin.	Kramer et al. 1985
**Neonatal/adult skin fibroblasts	**Laminin, type IV collagen, perlecan, nidogen/entactin and keratinocyte growth factor (KGF).	Woodley et al. 1988; Fleischmajer et al. 1988
*Epidermal keratinocytes	*Type IV procollagen, type IV & VII collagen, laminin, fibronectin and vascular endothelial growth factor/vascular permeability factor (VEGF/VPF).	O'Keefe et al. 1984; Petersen et al. 1988; Stern and Malhotra 1992; Ballaun et al. 1995

Microvascular endothelial cells isolated from the dermis of neonatal and adult skin (Kramer et al 1985) additionally secrete laminin. Furthermore, other reports suggested that newborn HDMECs shared with HUVECs the following properties:

- (1) Capacity to bind T-cells after IL-1 stimulation (Fleck et al. 1986).
- (2) To express Class II antigens after gamma-IF stimulation (Haskard et al. 1987).
- (3) To present Class II antigens to unprimed allogenic CD4⁺ T-cells (Sontheimer

1989).

Both dermal fibroblasts and epidermal keratinocytes secrete laminin and type IV collagen. This degree of commonality with human endothelial cell secretions suggested structural relatedness among ECMs.

Consequently, the approach adopted in this research study proposes a relatively non-immunogenic acellular ECM 'sandwich' assembly to act as a dermal wound repair template for the attraction, promotion of migration, and colonization by the host's cells, but more importantly, to allow the natural cellular formulation of the subsequent architecture of the healing tissue. In the finished product, this assembly would consist of a gelatin-fibronectin-heparan sulfate gel base which would be overlaid in turn by HUVECs ECM followed by a mixed matrix ECM secreted by HDMECs and dermal fibroblasts.

This study was attempted to determine whether epidermal keratinocytes would adhere and proliferate normally on either HUVECs ECM/gelatin or the mixed matrix overlaid on HUVECs ECM/gelatin. Furthermore, whether heterologous dermal fibroblasts and HDMECs would also adhere and proliferate on either of the prepared substrates. If affirmative, these results would endow a degree of credibility on the proposed acellular scaffold.

Materials and methods

Cell Culture

T-25 and T-75 tissue culture flasks were bought from Corning, NY. Falcon multi-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ), Costar 6-well clusters, with and without inserts (#3450-clear, 0.4 μ m pore size (Costar Corporation, Cambridge,

MA) were used. Gelatin (lot #70K2369) Type B from bovine skin (Sigma Chemical Co., St. Louis, MO) was employed for coating culture plates, culture inserts and flasks (0.2% w/v in DPBS, pH 7.4). Fibronectin, heparan sulfate, solubilized Amphotericin B, collagenase Type I and ethylene diamine tetra-acetic acid (EDTA) were bought from Sigma Chemical Co., St. Louis, MO. Dispase was obtained from Collaborative Research, Walham, MA. and DiI-Ac-LDL from Biomedical Technologies, Inc., Stoughton, MA. Fetal bovine serum (FBS), Medium 199 (with Earle's salts, L-glutamine, and 2.2 mg/L sodium bicarbonate), Iscove's modified Dulbecco's medium (IMDM), Dulbecco's phosphate buffered saline (DPBS) with and without calcium and magnesium, 0.05% trypsin/0.02% EDTA, soybean trypsin inhibitor, penicillin/streptomycin were obtained from Gibco Life Technologies, Grand Island, NY. EpiLife® medium and EpiLife® defined growth supplement, EDGS lot # ISO105 were purchased from Cascade Biologics, Inc. Portland, OR.

EDGS consists bovine serum albumin 95% fatty acid free, bovine transferrin, recombinant IGF-1, recombinant EGF, hydrocortisone, PGE-2 (private communication, Cascade Biologics, Inc.).

Keratinocytes

Primary adult epidermal keratinocytes (HEKa) at passage number one (P₁) were obtained from Cascade Biologics, Inc. Portland, OR. Cultures were maintained in serum-free EpiLife medium containing 0.06mM CaCl₂ in T-25 flasks in the Department of Dermatology and Cutaneous Surgery. 80% confluence was routinely reached in 5-7 days. Sub-culturing at a 3:1 split ratio was achieved with trypsin/EDTA, and an equivalent volume of soybean trypsin inhibitor was added. Centrifugation was followed by

resuspension in EpiLife medium, supplemented with EDGS. Since the purchased keratinocytes were guaranteed for 14 population doublings, and previous experience with these cell lines had shown retention of phenotypic characteristics, they were used at P₆. Routine seeding density was 1.2×10^5 cells.

HUVECs

HUVECs were prepared (Jaffe et al. 1973) from single umbilical cords, (4, in total) after proper donor screening for negative HIV, syphilis, and hepatitis B, C. The cords were gifts from the Maternity Unit of Jackson Memorial Hospital/University of Miami School of Medicine. Cells from single cords were passaged at a 1:2 split ratio with trypsin/EDTA or with 5mM EDTA/DPBS (pH 7.4) and used at passage numbers 2-6. Cell lines were maintained in complete Medium 199 with 20% FBS, 1% (v/v) EDGS and 100 U/ml penicillin and 100 µg/ml streptomycin in gelatin-coated T-25 flasks. A Nikon phase-contrast microscope was used throughout this study.

Preparation of HUVECs ECM (n=24)

HUVECs ECM was prepared as described previously (Solomon 1992). Aliquots of 5mM EDTA/DPBS (pH 7.4) were added to post-confluent layers of HUVECs, earlier seeded onto gelatin-coated culture inserts, 6-well culture plates, and T-25 flasks until all HUVECs had lifted off and acellular surfaces were left behind. The final aliquot of 5mM EDTA/DPBS was left in contact with the almost acellular ECM overnight in an attempt to inactivate any residual ECM proteolytic activity (Korner et al. 1993). Thorough rinsing with DPBS followed. Retracted HUVECs were immersed in complete Medium 199, centrifuged, resuspended and reseeded onto gelatin-coated flasks for further propagation. Unused ECMs were covered with DPBS (pH 7.4), and stored in the incubator in 5%

CO₂/95% O₂ at 37°C. They were usable for four weeks thereafter.

Seeding of epidermal keratinocytes on HUVECs ECM (n=8)

Trypsinized normal adult keratinocytes in EpiLife medium were seeded onto the HUVECs ECM in cell inserts. Costar #3450 6-well clusters were used. Seeding density was 1.2×10^5 cells.

Preparation and examination of adult epidermis after Dispase digestion (n=42)

Full thickness skin samples (4, in total) from pre-screened cadaver tissue donors (permission for research was obtained from next-of-kin) and fresh skin tissue samples (3, in total) were immersed in saline/antibiotic solution. After washing with DPBS containing penicillin/streptomycin and Amphotericin B, subcutaneous fat was removed, followed by dissection into 25mm² pieces. From each skin tissue sample (fresh or cadaver), 6-25mm² segments were dissected. Each segment was laid epidermis-side down over a small pond of IMDM (or Medium 199) containing 2% Dispase contained in each well of a 6-well culture plate, and incubated at 37°C for 16 hours. On the following morning, extensive rinsing in complete IMDM (or Medium 199) containing 20% FBS, penicillin/streptomycin and Amphotericin B was followed by more rinsing in DPBS (pH 7.4). A pair of small forceps was used to strip the epidermis.

Strips of epidermis were placed in DPBS and examined. There were small sections devoid of epidermal keratinocytes and grayish-white cuboidal 'chain link' areas could be seen, indicative of the epidermal-dermal junction. Where punctured, an underlying layer of papillary dermis consisting of tightly packed rounded cells could be clearly discerned.

Preparation of a mixed culture of HDMECs and dermal fibroblasts (n=12)

After removal of the epidermis, the exposed dermis was mechanically scraped with a

scalpel blade and abraded material was shaken off by swirling in a small amount of IMDM, (or Medium 199) containing 20% FBS with penicillin/streptomycin and Amphotericin B. The effluent was layered over HUVECs ECM, a gelatin-coated substrate, and plastic in T-25 flasks or 6-well culture plates. After 3 hours, nonattached material was removed, the cell layer was washed with DPBS and complete IMDM (or Medium 199) was added.

Using the mixed matrix from HDMECs/dermal fibroblasts as a substrate for (1)seeding keratinocytes (2) heterologous HDMECs and dermal fibroblasts (n=6)

Aliquots of 5mM EDTA/DPBS (pH 7.4) normally used in the preparation of acellular endothelial ECMs were used to obtain the mixed matrix from HDMECs/dermal fibroblasts proliferating on HUVECs ECM, gelatin or plastic. The two types of cells were morphologically recognized by their configurations in small areas of confluence and subjected to cell specific identity tests (e.g. DiI-Ac-LDL uptake for HDMECs). On all substrates, all cells rounded and lifted off within 45 minutes. These cells were pipetted into complete IMDM (or Medium 199), centrifuged and reseeded in complete IMDM, Medium 199 or HUVECs CM on HUVECs ECM, gelatin or plastic for further growth. The mixed matrix left behind was rinsed with DPBS and stored in the incubator. Trypsinized epidermal keratinocytes were seeded onto the mixed matrix. In other experiments, heterologous HDMECs and dermal fibroblasts were seeded.

Results

A number of HUVECs cell lines were grown from different umbilical cords, using the soluble growth factor, EDGS sold specifically for epidermal keratinocyte tissue culture in a serum-free medium. Keratinocytes, HDMECs and dermal fibroblasts adhered to the

HUVECs ECM substrate and the mixed matrix. Epidermal keratinocytes grown in serum free EpiLife medium did need EpiLife's EDGS growth supplement for optimal growth.

Attachment of trypsinized keratinocytes after seeding was rapid and timed at twenty minutes (HUVECs ECM>>gelatin>plastic) and proliferation proceeded with normal phenotypic morphology (Fig. 1).

Twenty-four hours post-seeding, there was a striking difference between cells seeded on HUVECs ECM and those seeded on gelatin. The former showed a higher percentage of adhesion and a 50% faster growth rate, calculated as number of days from adhesion to confluence. HUVECs ECM prepared from four different cell lines, from different passage numbers, or stored in the incubator for varying periods up to four weeks, did not alter the timed period of attachment of trypsinized keratinocytes. The same results were obtained with the mixed matrix. There was no evidence of multilayer keratinocyte formation in any of these experiments. The cell population on HUVECs ECM and mixed matrix increased by a factor of 10 ($1.2 \times 10^5 \rightarrow 1.2 \times 10^6$ cells) within 5 days.

After examination intact strips of epidermis or dissected portions were removed to wells (with the epidermal-side downward) containing 2ml of EpiLife medium, complete IMDM (or Medium 199) and incubated. Early on in this prolonged incubation, the epidermal keratinocyte layers (appearing to be dark brown under phase-contrast microscopy) folded and curled, large and small colonies of keratinocytes detached, appearing as brown rosettes. More and more of the grayish-white epidermal-dermal junction was exposed, until it was devoid of keratinocytes. Few colonies attached to plastic, gelatin or HUVECs ECM over a two-month period. HUVECs CM containing secreted type IV procollagen, and fibronectin (as well as thrombospondin-see

Introduction) was deemed an eminently suitable neutralizing agent for Dispase which is both a Type IV collagenase and a fibronectinase. Subsequently, on HUVECs ECM, mixed matrix, gelatin and plastic substrates, 50% EpiLife medium: 50% HUVECs CM, 100% HUVECs CM and 200 μ l HUVECs CM: 3ml EpiLife medium were tried in an attempt to achieve attachment. Attachment was achieved after one week's undisturbed incubation with 200ul HUVECs CM: 3ml EpiLife medium, 4-5 days with the 50/50 mixture, and 1-3 days with 100% HUVECs CM.

The brown rosettes of epidermal keratinocytes adhered to the HUVECs ECM, mixed matrix, gelatin or plastic and epidermal cells outgrew concentrically in islands (Fig.2). After two weeks incubation, these epidermal islands lifted off plastic substrates, but remained structurally intact. The circumference of the islands on plastic was always considerably smaller than those on HUVECs ECM, mixed matrix and gelatin. There was no cellular debris or any other sign of culture distress. HDMECs did not form tube like structures. The island of epidermal cells could be easily removed with a rubber policeman and reseeded elsewhere.

In subsequent experiments, Dispase-digested skin tissue segments were immersed in DPBS containing penicillin/streptomycin and Amphotericin B for 2 hours. Epidermis was stripped and inserted into wells of a culture plate containing HUVECs CM and incubated. Brown rosettes of keratinocytes were observed to adhere to plastic, gelatin, HUVECs ECM and mixed matrix after overnight incubation and during the next 2-3 days.

In the preparation of a mixed culture of HDMECs and dermal fibroblasts on HUVECs ECM, gelatin and plastic substrates, using Medium 199 or IMDM, outgrowth of a variety of different dermal cell types were morphologically identified, namely HDMECs, dermal

fibroblasts, melanocytes, dendrocytes. In contrast, when HUVECs CM was used only dermal fibroblasts and HDMECs adhered (Fig. 3). Later experiments using HUVECS CM as the starting medium yielded similar results after 3 hours incubation on both gelatin and plastic substrates. In the mixed culture of HDMECs and dermal fibroblasts grown on HUVECs ECM, gelatin or plastic, no soluble growth factor was added since dermal fibroblasts produce basic fibroblast growth factor (b-FGF). Heterologous HDMECs and fibroblasts in complete Medium 199, IMDM or HUVECs CM did not attach as quickly to the mixed matrix as the trypsinized keratinocytes (3 hours compared to 20 minutes). There was a similar significant difference in growth rate (as was observed previously with HDMECs and dermal fibroblasts grown on HUVECs ECM) between cells seeded on the mixed matrix (Fig. 4), gelatin or plastic (mixed matrix>>gelatin> plastic). Gelatin or plastic substrates with HUVECs CM as the starting medium yielded a similar adhesion time of 3 hours.

Discussion

The consolidation of past scientific literature reports on described extracellular secretions from human endothelial cells, dermal fibroblasts and epidermal keratinocytes led to these studies. The first piece of *in vitro* experimental proof leading from similarity in extracellular secretions, was provided by the routine growth and subculture of different cell lines of HUVECs, a human endothelial cell line, using a commercially available keratinocyte growth factor, EDGS. The culture medium did not contain any recognized endothelial cell mitogen nor did EDGS whose constituents are fully described under Materials and methods.

5mM EDTA/DPBS (pH 7.4) was used to prepare endothelial ECMs in these experiments

because a basic tenet of in vitro keratinocyte proliferation is low calcium concentration. High calcium concentration promotes keratinocyte differentiation. In the preparation of endothelial ECMs, others have used 20mM ethylene glycol-bis-(β -aminoethyl ether)-N, N, N¹, N¹-tetra-acetic acid (EGTA) or 0.5% sodium deoxycholate (Herman and Castellot 1987); Triton X-100 and 20 mM NH₄OH in PBS or 2M urea in DMEM (Korner et al. 1993); 100mM NH₄OH (Sakariassen et al. 1983). When harsh chemical conditions are used, there is a questionable lack of assurance about the intactness of the prepared ECM. In all ECM preparations reported here, detached cells were immersed in complete Medium 199 or IMDM, centrifuged, resuspended and then reseeded for further growth and comparative observations with the previous flask of lower passage number.

Trypsin used in the passaging of keratinocytes breaks down intercellular junctions and does not interfere with the pattern of in vitro integrin receptor expression on the basal side of the keratinocytes. The finding that trypsinized epidermal keratinocytes easily adhered to HUVECs ECM and the mixed matrix secreted by HDMECs and dermal fibroblasts revealed the existence of a crossover tissue interaction with concomitant integrin versatility. These epidermal-mesenchymal inter-relationships were previously thought to be tissue-specific with importance in adult healing and embryological development (Kangesu 2001). Initial keratinocyte adhesion to HUVECs ECM must have involved cell-matrix recognition by basally localized integrin receptors on the epidermal keratinocytes, allied with the later presentation of focal adhesions (Yusko et al 2001). Meredith et al. 1993 were the first to report that cell adhesion to the ECM prevented apoptosis and involved an integrin modulation.

The question arising (to be answered in further studies) is whether the mode of

preparation of the HUVECs ECM or the mixed matrix (prepared from HDMECs/dermal fibroblasts) caused the activation of integrin receptors or effected the integrin expression on the acellular matrix.

A different mechanism excluding ECM participation was involved in the initial adhesion and proliferation of occasional brown rosettes amidst HDMECs and dermal fibroblasts, which readily occurred even on plastic substrates. The observation that intact epidermal islands lifted off from all four (HUVECs ECM, mixed matrix, gelatin and plastic) substrates suggested that a proliferation limit had been reached. An alternative explanation could be that on HUVECs ECM, mixed matrix and gelatin substrates, a point was reached (much later than on plastic) when the cultured epidermal keratinocytes in the presence of both fibroblasts and ECM proteins could no longer synthesize and secrete Type IV procollagen, which they could do in their absence (Petersen et al. 1988). This could explain the much smaller circumference of the epidermal islands lifting off from plastic substrates.

A number of reasons have been cited in the scientific literature for the less than 50% 'take' of cultured epithelial autografts (CEAs) to wound beds including loss of integrins and concomitant adhesion properties following Dispase detachment (Rennekampff et al. 1996a). Dispase, a neutral protease is both a fibronectinase and type IV collagenase (Stenn et al. 1989), dissolving the attachments between the basal keratinocytes and the basement membrane, without disturbing the desmosomal intercellular junctions between adjacent cells (Green 1991). However, it internalizes basal cell adhesion dependent domains containing the $\alpha 6 \beta 4$ integrin (Poumay et al. 1992), which is a receptor for laminin-5, an ECM component.

As was noted in the Introduction, the extracellular secretions of HUVECs include type IV procollagen, fibronectin, and thrombospondin. Therefore HUVECs CM was deemed a suitable neutralizing agent for Dispase and this premise was proved. Its suitability as a growth medium for epidermal keratinocytes, HDMECs and dermal fibroblasts was checked by constant repetition of new cultures and subcultures of all three types of cell. A redesign of CEAs incorporating the mixed matrix in their manufacture can be contemplated. The conclusion to be drawn from these studies is that the proposed acellular scaffold composed in part of a 'sandwich' of two ECMs (with the mixed matrix uppermost) might be viable as a structural substitute for the lamina densa and lamina lucida, damaged or completely missing in some wounds and body burns. It might also be useful as an alloplast in cosmetic and reconstructive plastic surgery. If *in vivo* extracellular secretions differ from those *in vitro*, then the premise fails. The main finding that cells of epithelial and mesenchymal origins share common attachment molecules, so called integrin versatility requires further study.

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